REMARKS/ARGUMENTS

Claims 1-4, 7, 9-19 and 41-44 are pending in this Application. The Office Action mailed on September 18, 2006, includes the following rejections:

1. Claims 1-4, 7, 9-19 and 41-44 are rejected under 35 U.S.C. § 112 first paragraph.
2. Claims 1-4, 7, 9-19 and 41-44 are rejected under 35 U.S.C. § 112 second paragraph.
3. Claims 15-17 and 19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Livesey, et al.
4. Claims 15-19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Dennis, et al.
5. Claims 15-19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Gulati, et al.
6. Claims 15-19 are rejected under 35 U.S.C. § 102(e) as being anticipated by Tanagho, et al.
7. Claims 15-19 are rejected under 35 U.S.C. § 102(e) as being anticipated by Atala.
8. Claims 1-3, 9-14 and 17 are rejected under 35 U.S.C. § 103.
9. Claims 4, 7 and 18 are rejected under 35 U.S.C. § 103.

Applicants respectfully address the basis for each of the Action's rejections below.

Support for the amendments to the claims can be found throughout the application. Evidence of the reduced immunogenic response may be found throughout the application. The specification supports the amendments to the claim 1, specifically paragraph [0022], which compares the acellular replacement tissue of the present invention to an allograft and shows a significantly reduced immunologic response because surface cell antigens have been removed. Paragraphs [0037-0051] state the immune response of tissue prepared with the method of the present invention show that the native cell-free tissue adapts to its environment and is not rejected. Furthermore, the composition of the present invention (the native cell-free tissue) is not rejected as other tissue replacements or allografts. The specification evaluates the native-cell free tissues for immune response following implantation of a cell-free sciatic nerve graft under various conditions, e.g., Figures 2-3 and Table 1 of the present application. In addition, paragraph [0043] compares the present invention to the current clinical approach (i.e., the autograft) used for several types of tissue repair (e.g., nerve tissue repair) and evaluates the immunologic response and the degree of immunologic rejection after surgery.

The specification also supports the amendments to the claims 41 and 42, specifically paragraph [0011], which defines the basal laminae and endoneurium layer retain substantially the native extracellular

matrix structure as retaining the natural and generally original structure of the basal laminae and endoneurium layer. The cellular components are specifically removed without significant alteration of the natural extracellular structure of the native extracellular matrix (ECM). The structure is preserved (referred to as intact structural components), specifically, the basal laminae and endoneurium/endothelial layer retain their natural and generally original structure. In addition, paragraph [0036] defines the removal of cells without creating structural damage (thereby retaining extracellular matrix and essential components).

Claim Rejections – Claims 1-4, 7, 9-19 and 41-44 are rejected under 35 U.S.C. § 112.

The Action rejects claims 1-4, 7, 9-19 and 41-44 based on not complying with the written description requirement of 35 U.S.C. § 112. The Action contends that the Triton X-200 listed in the specification provides examples of non-ionic detergents. Applicants assert that the specification provides examples of <u>anionic</u> detergents including Triton X-200.

The specification as filed (page 12, paragraph [0042]) provides examples of anionic detergents including Triton X-200. Triton X-200 is in-fact an <u>anionic</u> detergent. The manufacturer's product information sheet (attached as Appendix A and incorporated herein) lists Triton X-200 as an <u>anionic</u> detergent. Similarly, the Sigma-Aldrich detergent product index (attached as Appendix B and incorporated herein) lists Triton X-200 as an <u>anionic</u> detergent. Therefore, the specification does provide specific examples of anionic detergents. These are described in a way that the skilled artisan would know the inventors had possession of the claimed invention and fully complied with 35 U.S.C. § 112 first paragraph. The claims also particularly point out and distinctly claim the invention and fully complied with 35 U.S.C. § 112 second paragraph.

As such, the specification satisfies the written description requirement under 35 U.S.C. § 112. For the reasons mentioned above, the Applicants respectfully request the withdrawal of the rejection under 35 U.S.C. § 112.

Claims rejected under 35 U.S.C. § 102(b) as being anticipated.

The structure, properties and characteristics of the product of the present invention are very different from the structure, properties and characteristics of the products disclosed in Livesey, Dennis, Gulati, Tanagho and Atala. When assessing the patentability of product-by-process claims over the prior art, the structure implied by the process steps must be considered, especially where the product can be defined by the process steps by which the product is made, or where the manufacturing process steps would be expected to impart distinctive structural characteristics to the final product. See, e.g., *In re*

Garnero, 412 F.2d 276, 279, 162 USPQ 221, 223 (CCPA 1979). The products of claim 1-4, 7, 9-19 and 41-44 can be defined by the process steps by which the products are made and the process steps create distinctive structural characteristics in the final products.

The steps and materials used to prepare the graft of the present invention and the grafts of the cited references are different in both structure and physical characteristics and as a result, each of the final products are different in both structure and physical characteristics. For example, the attached (see Appendix C and incorporated by reference herein) Tissue Engineering article pages 1641-1651 (Volume 10, Number 11/12, 2004) (hereafter referred to as "Hudson") illustrates the importance of maintaining the internal structure and extracellular matrix components of a nerve tissue graft and compares the different methods of manufacturing nerve grafts. Hudson compares (page 1642 methods and materials section) a nerve tissue graft made by treatment with SB-10 (referred to in Hudson as "OA"), a chemical treatment method using sodium deoxycholate similar to United States Patent Number 6,371,992 (referred to in Hudson as "Sondell") and a freeze thaw method (referred to in Hudson as "F-T").

Hudson provides in Figure 5 (page 1647) an image of the cross-sections of basal laminae visualized by laminin staining that compares the basal laminae after treatment with SB-10 (referred to in Hudson as "OA"), a chemical treatment method using sodium deoxycholate similar to United States Patent Number 6,371,992 (referred to in Hudson as "Sondell") and a freeze thaw method (referred to in Hudson as "F-T"). The different treatments produce different products with different structures and different characteristics. Figure 5 of Hudson shows the cross sections of the basal laminae (i.e., the rings) after treatment, illustrating the fresh nerve tissue (Figure 5a of Hudson) and the SB-10 treated nerve tissue (Figure 5b of Hudson) have intact basal laminaes. In contrast, the chemical treatment with sodium deoxycholate by Sondell disrupted the basal laminae (Figure 5d of Hudson).

The different treatments produce different products having different characteristics. For example, Figure 7 of Hudson is a graph that compares the capacity to support regeneration or the axon density at both 28 and 84 days, in a fresh graft, in an OA treated graft (i.e., the sample with SB-10), in a graft treated with sodium deoxycholate (as in Sondell) and in a graft treated by the freeze thaw method. Figure 7 of Hudson shows the highest axon density at both 28 and 84 days is seen in the OA treated graft (i.e., the sample with SB-10). A decrease in the axon density from 28 days to 84 days is seen for the sodium deoxycholate treated graft (as in Sondell) and the freeze thaw graft (F/T). Hudson states that the OA graft showed a 910% higher axon density compared to the freeze thaw graft and a 401% higher axon density compared to the sodium deoxycholate treated graft (Sondell) (page 1650).

The treatment with SB-10 (e.g., OA treated graft of Hudson) showed a high capacity to support

regeneration and maintained the extracellular matrix components and structure. In contrast, the graft prepared by the freeze thaw method showed a lower capacity to support regeneration and did not remove the cellular debris; similarly, the graft prepared by the sodium deoxycholate treatment (as in Sondell) showed a lower capacity to support regeneration and did not retain the extracellular matrix and structure (see page 1648 and Figure 7 of Hudson). In addition, the treatment with SB-10 (e.g., the OA treated graft of Hudson) did not show an increase in the CD8+ cells, and indicated that a rejection reaction was not present (page 1649 of Hudson). The process steps by which the products are made impart distinctive structures, characteristics and properties to the final product.

Therefore, products made by different methods (e.g., freeze thaw treatments, chemical treatments and the present invention) having different steps, mechanisms and reagents impart different characteristics and properties on their respective products. Thus, the products made by these processes cannot be identical products.

Claims 15-17 and 19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Livesey, et al.

Applicants disagree with the Action's analysis of U.S. Patent No. 5,336,616 to Livesey, et al., ("Livesey"), which is said to disclose the claimed invention. Livesey does not anticipate claims 15-17 and 19 of the present invention. Livesey does not disclose the limitations related to nerve tissue, structural integrity, the reduction in the immune response of the graft or the enhanced capacity for regeneration produced by the present invention. As such, Livesey simply cannot anticipate the present invention.

The process steps of the present invention and Livesey are different; and, as a result, the products defined by the processes are different. Livesey discloses a product that is made by a process using different chemical agents to produce a product that has different properties than the product of the present invention. The chemical agents disclosed by Livesey include Triton X-100, polyoxyethylene (20) sorbitan mono-oleate and polyoxyethylene (80) sorbitan mono-oleate (Tween 20 and 80) (c.9, ll.41-52) which are non-ionic and sodium deoxycholate, and sodium dodecyl sulfate (*id.*) which are anionic. After Livesey decellularizes the tissue, it is incubated in a cryopreservation solution and cryopreserved. Furthermore, Livesey does not disclose sulfobetaines alone or in combination with an anionic surface-active detergent.

Although chemicals can be lumped into broad categories of generally similar characteristics, it <u>cannot</u> be said that each individual chemical of that category is the same (e.g., all organic compounds can

be placed into one category-organics, but all organic compounds are not the same). Similarly, detergents are different characteristics (e.g., composition, structure, characteristics, charge, size, etc.) and as such their interaction with a substrate is different because it is dictated by the characteristics of the detergent. Although, some general characteristics of being a detergent may be shared, the individual compounds are not interchangeable as their specific compositions, structures, characteristics, charges and sizes are different.

The chemicals used in the present invention and Livesey have different structures, different chemical formulas and different characteristics; therefore, the products produced by these processes cannot be identical. The skilled artisan knows that different compounds have different properties (e.g., the critical micelle concentration value, solubility, amount of damage to protein structures, amount of myelin basic protein removed and so forth) and with different properties, the ability to decellularize tissue is different. As the degree of decellularization is different, the product must be different. With differences in the degree of decellularization, the present invention and the product of Livesey cannot be identical. Therefore, the process of the present invention imparts distinctive structural characteristics to the final product.

Applicants respectfully submit that the Livesey fails to meet the standard of 35 U.S.C. § 102(b). As such, Livesey does not anticipate any of the claims of the present invention. Applicants respectfully request the withdrawal of the rejection under 35 U.S.C. §102(b).

Claims 15-19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Dennis, et al.

The Action rejects claims 15-19 under 35 U.S.C. § 102(b) as anticipated by Dennis, et al., ("Dennis") (U.S. Patent No. 6,207,451), which is said to disclose the claimed invention. Applicants respectfully submit that the cited reference fails to meet the standard of 35 U.S.C. § 102(b).

The products of claim 15-19 can be defined by the process steps by which the products are made and these process steps impart distinctive structural characteristics to the final product. The process steps of the present invention and Dennis are different and, as a result, the products defined by the processes are different. Dennis teaches acellularized muscle anchors made by removing the muscles tissue from a subject, cut the muscles tissue into strips and pinned them to a substrate. The muscle strips are treated with a NaN_3 solution a deoxycholic acid (sodium salt), a solution of SDS and a solution of TRITON X-100.

Dennis teaches a product made by a process using mammalian muscle construct, which is developed *in vitro* from cells extracted from mammals. First, Dennis relates to muscle tissue. Second, the

product made by the process of Dennis and the product made by the present invention are different. Third, Dennis does not disclose the treatment with one or more sulfobetaines, nor does Dennis disclose the treatment with sulfobetaines and an anionic surface-active detergent. Dennis and the present invention use very different processes and reagents. As a result these differences impart distinctive structural characteristics to the respective final products (for the same reason as stated above).

Applicants respectfully submit that Dennis fails to meet the standard of 35 U.S.C. § 102(b). The products of the present invention can be defined by the process steps by which they are made and those process steps impart distinctive structural characteristics to the final products. As such Dennis does not anticipate any of the claims of the present invention. Applicants respectfully request the withdrawal of the rejection under 35 U.S.C. §102(b).

Claims 15-19 are rejected under 35 U.S.C. § 102(b) as being anticipated by Gulati, et al.

The Action rejects claims 15-19 under 35 U.S.C. § 102(b) as anticipated by Gulati, et al., ("Gulati"), which is said to disclose the claimed invention. Applicants respectfully submit that the cited reference fails to meet the standard of 35 U.S.C. § 102(b).

The products of claim 15-19 of the present invention can be defined by the process steps by which the products are made and the process steps impart distinctive structural characteristics to the final product. The process steps of the present invention and Gulati are different; and, as a result, the products defined by the processes are different. Gulati discloses a product that is made by a process of harvesting degenerated nerve cells and <u>repeatedly freezing them in N_2 (l)</u>. Gulati then places the nerve cell on a dish of cultured cells (see page 120, section 2.3). It is unclear how a single nerve cell on an *in vitro* tissue culture that is repeatedly frozen and thawed is the same as a native, cell-free tissue replacement. Regardless, the process of Gulati creates a product that has a different composition, structure and characteristics than the product of the present invention.

Gulati does not disclose a tissue replacement made by soaking a tissue in a solution having one or more sulfobetaines, washing the tissue replacement in one or more solutions of a buffered salt, extracting with an anionic surface-active detergent and washing the tissue replacement in one or more solutions of a buffered salt. Gulati and the present invention are clearly different in processes and as such impart distinctive structural characteristics to the final product. Therefore, the product in Gulati and the product of the present invention are different, made by different processes and possess different characteristics.

Applicants respectfully submit that the Gulati fails to meet the standard of 35 U.S.C. § 102(b). As such Gulati does not anticipate any of the claims of the present invention. Applicants respectfully

request the withdrawal of the rejection under 35 U.S.C. §102(b).

Claims 15-19 are rejected under 35 U.S.C. § 102(e) as being anticipated by Tanagho, et al.

The Action rejects claims 15-19 under 35 U.S.C. § 102(b) as anticipated by Tanagho, et al., United States Patent Number 6,371,992 ("Tanagho"), which is said to disclose the claimed invention. Applicants respectfully submit that the cited reference fails to meet the standard of 35 U.S.C. § 102(e).

The process steps of the present invention and the process steps in Tanagho are different; and, as a result, the products made by these processes are different. Tanagho discloses a product that is made using a chemical treatment that includes a sodium deoxycholate solution containing sodium azide to remove cell membranes and intracellular lipids from the intermediate matrix. Tanagho <u>does not</u> disclose a nerve tissue replacement product obtained by a soaking an obtained nerve tissue replacement in a solution having one or more sulfobetaines, washing the tissue replacement in one or more solutions of a buffered salt, extracting with an anionic surface-active detergent and washing the tissue replacement in one or more solutions of a buffered salt.

The skilled artisan knows that different compounds have different properties (e.g., the critical micelle concentration value, solubility, amount of damage to protein structures, amount of myelin basic protein removed and so forth) and with different properties, the ability to decellularize tissue is different. As the degree of decellularization is different, the final product must be different, as shown in Figure 10. Therefore, the differences in the degree of decellularization between the product of the present invention and the product of Tanagho result in the products being different (e.g., having different compositions, structures and characteristics). The different properties of the compounds used in Tanagho and the present invention result in different components being removed from the tissue to form a product having a unique internal structure with different extracellular matrix (ECM) components. The process of the present invention imparts distinctive structural characteristics to the final product. Thus, the product of Tanagho does not maintain the same composition as the product of the present invention so they <u>cannot</u> be identical.

Applicants respectfully submit that the Tanagho fails to meet the standard of 35 U.S.C. § 102(e). As such Tanagho does not anticipate any of the claims of the present invention. Applicants respectfully request the withdrawal of the rejection under 35 U.S.C. §102(e).

Claim Rejections – Claims 15-19 are rejected under 35 U.S.C. § 102(e) as being anticipated by Atala.

The Action rejects claims 15-19 under 35 U.S.C. § 102(e) as anticipated by Atala, United States Patent Number 6,376,244 ("Atala"), which is said to disclose the claimed invention. Applicants

respectfully submit that the cited reference fails to meet the standard of 35 U.S.C. § 102(e).

First, Atala relates to an organ or part of an organ. Second, the process steps of the present invention and Atala are different and as a result, the products defined by those processes are different. Third, Atala discloses a product that is made using severe mechanical treatments using a magnetic stir plate and a paddle or a rotator platform. In contrast, the present invention provides a tissue replacement product obtained by soaking an obtained tissue replacement in a solution having one or more sulfobetaines, washing the tissue replacement in one or more solutions of a buffered salt, extracting with an anionic surface-active detergent and washing the tissue replacement in one or more solutions of a buffered salt. The process used in Atala and the present invention are different and impart different characteristics on the respective products. These distinctly different processes result in distinctly different final products. Thus, the product of Atala does not maintain the same composition as the product of the present invention so they <u>cannot</u> be identical.

Applicants respectfully submit that the Atala fails to meet the standard of 35 U.S.C. § 102(e). As such, Atala does not anticipate any of the claims of the present invention. Applicants respectfully request the withdrawal of the rejection under 35 U.S.C. §102(e).

Claims 1-3, 9-14 and 17 are rejected under 35 U.S.C. § 103 as being unpatentable over Livesey in view of "Detergent Properties and Applications"

Applicants respectfully submit that claims 1-3, 9-14 and 17 are not obvious over the cited art and are, therefore, allowable under 35 U.S.C. § 103(a) for the reasons stated below.

A prima facie case of obviousness has not been established as (1) the prior art or combined references does not teach or suggest all the claim limitations, (2) there is no reasonable expectation of success and (3) there is no suggestion or motivation in the prior art to modify the reference or to combine reference teachings as proposed.

The Action states it would have been obvious to combine Livesey with a reference entitled, "Detergent Properties and Applications" to achieve the present invention. Livesey as discussed, *supra* (arguments incorporated herein by reference) does not include each and every limitation of the present invention. Livesey does not disclose nerve tissue replacements, does not disclose treatment with sulfobetaines, and the product formed by Livesey is different than the product of the present invention. The "Detergent Properties and Applications" reference is merely a list lumping the zwitterionic detergents together; however, each of the detergents are different, each having different structures, characteristics and properties. The addition of the cited reference does not cure the deficiencies of Livesey, and even if the cited reference did (which it does not), a prima facie case of obviousness would still not established

because there is not a reasonable expectation of success and no suggestion or motivation in the prior art to modify the reference or to combine reference teachings as proposed.

Applicants assert that all zwitterionic detergents are not interchangeable and blindly/randomly selecting one from a list of compounds does not provide <u>any</u> reasonable expectation of success and neither Livesey, the cited reference or any combination thereof suggest or provide motivation or guidance as to which of the numerous compounds to select one from the list. (see *In re Ruschig et al.*, 145 U.S.P.Q. 274 (C.C.P.A. 1965) stating an anticipation is not made out through hindsight selection based on applicant's disclosure of variables of a broad generic disclosure.) For example, studies have compared CHAPS to SB-10 and SB-16, as well as numerous other detergents, covering the zwitterionic, anionic, cationic, and non-ionic categories and <u>significant</u> differences were seen in their affect on nerve tissue. Knowing how each of these factors will impact the various components within nerve tissue (e.g., myelin, axons, collagen, laminin) is by no means obvious or trivial.

In addition, the Action's statement that the use of de-ionized distilled water would have been obvious is incorrect. Livesey taught the use of de-ionized water to wash off the fascia. In contrast, distilled water loosens the myelin sheaths (which are about 90% lipid) that surround the axons and swells in the presence of distilled water and allows the subsequent detergent solutions to penetrate and disrupt the cellular membranes of the myelin sheaths.

Accordingly, Applicants respectfully submit that the claims are not obvious over Livesey and the Sigma-Aldrich reference "Detergent Properties and Applications" and are, therefore, allowable under 35 U.S.C. § 103(a). Applicants respectfully request that the rejection of the claims be withdrawn.

Claim Rejections – Claims 4, 7 and 18 are rejected under 35 U.S.C. § 103 as being unpatentable over Livesey in view of Atala

Applicants respectfully submit that claims 4, 7 and 18 are not obvious over the cited art and are, therefore, allowable under 35 U.S.C. § 103(a) for the reasons stated below.

Neither Livesey or Atala (each of which are discussed *supra* and arguments incorporate herein by reference) nor any combination thereof teach or suggest all the claim limitations. Furthermore, there is no reasonable expectation of success and there is no suggestion or motivation in the prior art to modify the reference or to combine reference teachings as proposed. As such, a prima facie case of obviousness has not been established. Applicants respectfully request that the rejection of claims 4, 7 and 18 be withdrawn.

Conclusion

In light of the remarks and arguments presented above, Applicants respectfully submit that the claims in the Application are in condition for allowance. Favorable consideration and allowance of the pending claims 1-4, 7, 9-19 and 41-44 are therefore respectfully requested.

Applicants believe no fees are due at this time. If the Examiner has any questions or comments, or if further clarification is required, it is requested that the Examiner contact the undersigned at the telephone number listed below.

Dated: July 11, 2007.

Respectfully submitted,

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Appendix A

Product Information

DOW Surfactants

Page 1 of 2

TRITON* X-200 Surfactant

Benefits
- Excellent detergent with lathering properties
- High stable foam
- Low toxicity
- Effective in hard water
- Good stability to electrolytes & chlorine

Applications
- Personal care applications
- Emulsion polymerization
- Mild alkaline cleaners
- Heavy duty cleaners

Physical Properties

Actives, wt%	28
Solvent	Water
Appearance	Opaque, white liquid
pH, 5% aq solution	6.6
Viscosity at 25°C (77°F), cP	7000
Density at 25°C (77°F), g/mL	1.068
Flash Pt, Closed Cup, ASTM D93	None
Pour point, °C (°F)	-1 (30)

NOTE: Additional physical and chemical property data is located on the product Material Safety Data Sheet.

Performance Properties

Equilibrium surface tension[1], dynes/cm	30
Critical micelle concentration in distilled water at 25°C (77°F), ppm	970
Draves 25 sec wetting conc. wt% at 25°C (77°F)	0.07
Ross-Miles Foam Test, Initial/5 min, 0.1% at 25°C (77°F), mm	88/81
50°C (122°F)	155/75

[1]Measured at 0.1 wt% and 25 °C (77 °F)

Solubility and Compatibility
- Soluble in water
- Chemically stable in acids & bases
- Compatible with nonionic & other anionic detergents

Chemical Description
Name: Polyether sulfonate
Surfactant Type: Anionic

Additional product information and performance data is available by requesting datasheets that are listed on the backside of this page.

Appendix A

DOW Surfactants
Page 2 of 2

Datasheets

- DOW Specialty Surfactants Reference Chart, 119-01491
- TRITON & TERGITOL Surfactants for Household, Industrial & Institutional Cleaning CD, 119-01466-0501
- TRITON & TERGITOL Surfactants for Paint, Coatings, Adhesives, Stabilizers & Emulsion Polymerization CD, 119-01536
- Contact DOW Customer Service for current listing on conformance of TRITON Surfactants with U. S. FDA Regulations

Appendix B

Copy of information on the Sigma-Aldrich website listing of products classified by properties of detergents, e.g., Anionic and non-ionic.

SIGMA-ALDRICH

Register for additional site benefits. | Login | Your Profile | Order Center | Search | Oligos & Peptides | Support | Help

- Antibiotic Explorer
- Biological Buffers
- Biological Detergents
  - Detergent Product Index
  - Key Resources
- Books
- Enzyme Explorer
- Lipids
- Peptide Explorer
- Pharmacopoeia
- PolyAmino Acids
- Radiochemicals
- Vitamins and Derivatives

Detergent Product Index

Biological Detergents

- Anionic
- Anti-Foaming Agent
- Cationic
- Non-Ionic
- Zwitterionic

Anionic

Product Name	Product #
Chenodeoxycholic acid, minimum 98%	C9377
Chenodeoxycholic acid sodium salt, minimum 97%	C8261
Cholic acid, ox or sheep bile, minimum 98%	C1129
Dehydrocholic acid	D3750
Deoxycholic acid, minimum 99%	D2510
Deoxycholic acid, SigmaUltra, minimum 99%	D4297
Deoxycholic acid methyl ester, approx. 98%	D3761
Digitonin, Solid	D141
Digitonin, cholesterol determination, approx. 50% (TLC)	D5628
Digitoxigenin	D9404
N,N-Dimethyldodecylamine N-oxide	D3775
Docusate sodium salt, minimum 99%, Waxy solid	D0885
Docusate sodium salt, SigmaUltra, minimum 99%	D4422
Glycochenodeoxycholic acid sodium salt, minimum 97% (TLC)	G0759
Glycocholic acid hydrate, synthetic, minimum 97% (TLC)	G2878
Glycocholic acid sodium salt hydrate, synthetic, minimum 97% (TLC)	G7132
Glycodeoxycholic acid monohydrate, minimum 97% (TLC)	G6132
Glycodeoxycholic acid sodium salt, minimum 97%	G3258
Glycodeoxycholic acid sodium salt, SigmaUltra, minimum 97% (TLC)	G9910
Glycolithocholic acid 3-sulfate disodium salt	G6282
Glycolithocholic acid ethyl ester	G7640
N-Lauroylsarcosine sodium salt, minimum 94%	L5125
N-Lauroylsarcosine sodium salt, SigmaUltra, minimum 97%	L5777
N-Lauroylsarcosine solution, minimum 95%	L9038
N-Lauroylsarcosine solution, 20%	L7414
Lithium dodecyl sulfate, for electrophoresis, denatured polyacrylamide gel electrophoresis, especially at lower temperature conditions, approx. 99% (GC)	L2274
Lithium dodecyl sulfate, minimum 98.5% (GC)	L4632
Lithium dodecyl sulfate, SigmaUltra, >99% (GC)	L5901
Lugol solution	L6146
Niaproof 4, Type 4, approx. 27%	4
Niaproof 4, SigmaUltra, Type 4, approx. 27%	N1404
Triton QS-15	QS-15
Triton QS-44 solution, 80% in water	QS-44

Appendix B

1-Octanesulfonic acid sodium salt, SigmaUltra	O0133
1-Octanesulfonic acid sodium salt, approx. 98%	O8380
Sodium 1-butanesulfonate	B2013
Sodium 1-decanesulfonate, SigmaUltra, approx. 98%	D0181
Sodium 1-decanesulfonate, approx. 98%	D3412
Sodium 1-dodecanesulfonate	D5266
Sodium 1-heptanesulfonate anhydrous, Solid	H2766
Sodium 1-heptanesulfonate anhydrous, SigmaUltra	H8901
Sodium 1-nonanesulfonate, approx. 98%	N0893
Sodium 1-propanesulfonate monohydrate	P4809
Sodium 2-bromoethanesulfonate, minimum 98%	B9638
Sodium cholate hydrate, ox or sheep bile, minimum 99%	C1254
Sodium cholate hydrate, SigmaUltra, minimum 99%	C6445
Sodium choleate	S9875
Sodium deoxycholate, minimum 97%	D5760
Sodium deoxycholate monohydrate, SigmaUltra, >99% (titration)	D5670
Sodium dodecyl sulfate, minimum 98.5% (GC)	L4509
Sodium dodecyl sulfate, approx. 95% as based on total alkyl sulfate content	L5750
Sodium dodecyl sulfate, SigmaUltra, >99% (GC)	L6026
Sodium hexanesulfonate anhydrous, approx. 98%	H5263
Sodium hexanesulfonate anhydrous, SigmaUltra	H8025
Sodium octyl sulfate, approx. 95%	O4003
Sodium pentanesulfonate anhydrous	P0259
Sodium pentanesulfonate anhydrous, SigmaUltra	P8159
Sodium taurocholate, ox bile	T0750
Sodium taurocholate, minimum 97% (TLC)	T4009
Taurochenodeoxycholic acid sodium salt	T6260
Taurodeoxycholic acid sodium salt monohydrate, SigmaUltra, minimum 97% (TLC)	T0557
Taurodeoxycholic acid sodium salt monohydrate, minimum 97% (TLC)	T0875
Taurohyodeoxycholic acid sodium salt hydrate, minimum 98%	T0682
Taurolithocholic acid 3-sulfate disodium salt	T0512
Tauroursodeoxycholic acid sodium salt, approx. 96%	T0266
Triton X-200 solution, 28% in water (dispersion)	X-200
Triton® XQS-20 solution	XQS20
Trizma® dodecyl sulfate	L5649
Ursodeoxycholic acid, minimum 99%	U5127

Anti-Foaming Agent

back to top

Product Name	Product #
Antifoam 204	A6426
Antifoam A Concentrate	A5633
Antifoam A Emulsion	A5758
Antifoam B Emulsion	A5757
Antifoam C Emulsion	A8011

Cationic

back to top

Product Name	Product #
Alkyltrimethylammonium bromide	M7635
Benzalkonium chloride, Semisolid	B1383

Appendix B

Detergent Product Index for Anionic, Anti-Foaming Agents, Cationic, and Zwitterionic detergents

Benzalkonium chloride, SigmaUltra	B6395
Benzyldimethylhexadecylammonium chloride	B4136
Benzyldimethyltetradecylammonium chloride	B5651
Benzyldodecyldimethylammonium bromide	B5776
Benzyltrimethylammonium tetrachloroiodate, minimum 95% (titration)	B5042
Dimethyldioctadecylammonium bromide	D2779
Dodecylethyldimethylammonium bromide	D1794
Dodecyltrimethylammonium bromide, SigmaUltra, approx. 99%	D5047
Dodecyltrimethylammonium bromide, approx. 99%	D8638
Ethylhexadecyldimethylammonium bromide	E6633
Girard's reagent T, Crystalline	G1767
Hexadecyltrimethylammonium bromide, minimum 99%, Powder	H5882
Hexadecyltrimethylammonium bromide, SigmaUltra, approx. 99%	H9151
N,N',N'-Polyoxyethylene(10)-N-tallow-1,3-diaminopropane, Liquid	P4162
Thonzonium bromide	T7783
Trimethyl(tetradecyl)ammonium bromide, approx. 99%	T4762

Non-Ionic back to top

Product Name	Product #
BigCHAP, approx. 98%	B2518
Bis(polyethylene glycol bis[imidazoyl carbonyl]), Powder	P9632
Brij® 35, Stein-Moore chromatography	P1254
Brij® 56	P5759
Brij® 72	P3684
Brij® 76	P5516
Brij® 92V	P4144
Brij® 97	P5136
Brij® 58P	P5884
Cremophor® EL	C5135
Decaethylene glycol monododecyl ether	P9769
N-Decanoyl-N-methylglucamine, minimum 98%	D6277
n-Decyl α-D-glucopyranoside, approx. 98% (GC)	D5419
Decyl β-D-maltopyranoside, minimum 98% (GC)	D7658
n-Dodecanoyl-N-methylglucamide, approx. 98%	D4191
n-Dodecyl α-D-maltoside, minimum 95% (GC)	D5644
n-Dodecyl β-D-maltoside, minimum 98%	D4641
n-Dodecyl β-D-maltoside, SigmaUltra, minimum 98% (GC)	D5172
Heptaethylene glycol monodecyl ether, minimum 97% (GC)	P7880
Heptaethylene glycol monododecyl ether	P9800
Heptaethylene glycol monotetradecyl ether	P9675
n-Hexadecyl β-D-maltoside, minimum 98% (GC)	H6392
Hexaethylene glycol monododecyl ether	P9875
Hexaethylene glycol monohexadecyl ether	P0428
Hexaethylene glycol monooctadecyl ether	P1178
Hexaethylene glycol monotetradecyl ether	P5550
Igepal CA-630, Viscous liquid	I3021
Igepal CA-630, for electrophoresis, suitable 2-D electrophoresis	I7771
Methyl-6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside, minimum 98% (TLC)	M3430

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Appendix B

Detergent Product Index for Anionic, Anti-Foaming Agents, Cationic, and Zwitterionic detergents

Nonaethylene glycol monododecyl ether	P9641
N-Nonanoyl-N-methylglucamine, approx. 98%	N1138
N-Nonanoyl-N-methylglucamine, SigmaUltra, approx. 98%	N3030
Octaethylene glycol monodecyl ether	P7925
Octaethylene glycol monododecyl ether	P8925
Octaethylene glycol monohexadecyl ether	P5678
Octaethylene glycol monooctadecyl ether	P1429
Octaethylene glycol monotetradecyl ether	P9680
Octyl-β-D-glucopyranoside, minimum 98%	O8001
Pentaethylene glycol monodecyl ether, minimum 97%	P7430
Pentaethylene glycol monododecyl ether	P8652
Pentaethylene glycol monohexadecyl ether	P3061
Pentaethylene glycol monohexyl ether, >95% (GC)	P4529
Pentaethylene glycol monooctadecyl ether	P1051
Pentaethylene glycol monooctyl ether	P4654
Polyethylene glycol diglycidyl ether, approx. 75%	P2672
Polyethylene glycol ether W-1	P7516
Polyoxyethylene 10 tridecyl ether	P2393
Polyoxyethylene 100 stearate	P3690
Polyoxyethylene 20 isohexadecyl ether	P3315
Polyoxyethylene 20 oleyl ether	P5641
Polyoxyethylene 40 stearate	P3440
Polyoxyethylene 50 stearate	P3565
Polyoxyethylene 8 stearate	P3015
Polyoxyethylene bis(imidazolyl carbonyl)	P5602
Polyoxyethylene 25 propylene glycol stearate	P2440
Saponin from Quillaja bark	S2149
Saponin from Quillaja bark	S4521
Saponin from Quillaja bark	S7900
Span® 20	S6635
Span® 40	S6635
Span® 60	S7010
Span® 65	S3026
Span® 80	S6760
Span® 85	S7135
Tergitol, Type 15-S-12	15-S-12
Tergitol, Type 15-S-30	15-S-30
Tergitol, Type 15-S-5	15-S-5
Tergitol, Type 15-S-7	15-S-7
Tergitol, Type 15-S-9	15-S-9
Tergitol, Type NP-10	NP-10
Tergitol, Type NP-4	NP-4
Tergitol, Type NP-40	NP-40
Tergitol, Type NP-7	NP-7
Tergitol, Type NP-9	NP-9
Tergitol, MIN FOAM 1x	T1135
Tergitol, MIN FOAM 2x	T1360

Appendix B

Detergent Product Index for Anionic, Anti-Foaming Agents, Cationic, and Zwitterionic detergents

Tergitol, Type TMN-10	TMN-10
Tergitol, Type TMN-6	TMN-6
Tetradecyl-β-D-maltoside, minimum 98% (GC)	T3524
Tetraethylene glycol monodecyl ether, approx. 97% (GC)	P7425
Tetraethylene glycol monododecyl ether	P4391
Tetraethylene glycol monotetradecyl ether	P9300
Triethylene glycol monodecyl ether, minimum 97% (GC)	P7300
Triethylene glycol monododecyl ether	P8425
Triethylene glycol monohexadecyl ether	P9925
Triethylene glycol monooctyl ether, Liquid	T3789
Triethylene glycol monotetradecyl ether	P9175
Triton CF-21, Solution	CF-21
Triton CF-32, 95% in water	CF-32
Triton DF-12	DF-12
Triton DF-16	DF-16
Triton GR-5M, 90% in aqueous 20% isopropanol	GR-5M
Triton X-100, reduced	X-100-RS
Triton X-102	X-102
Triton X-15	X-15
Triton X-151 solution, 65% in water	X-151
Triton X-207	X-207
Triton® X-100, SigmaUltra	T9284
Triton® X-100, Peroxide- and carbonyl-free	X-100-PC
Triton® X-114	X-114
Triton® X-165 solution, 70% in water	X-165
Triton® X-305 solution, 70% in water	X-305
Triton® X-405 solution, 70% in water	X-405
Triton® X-45	X-45
Triton® X-705-70 solution, 70% in water	X705-70
TWEEN® 20, Viscous liquid	P1379
TWEEN® 20, Low-peroxide, Low-carbonyls	P6585
TWEEN® 20, SigmaUltra	P7949
TWEEN® 20, Low-peroxide, Low-carbonyls	P9341
TWEEN® 20 solution, 70% in water	P2860
TWEEN® 20 solution, 10% in water	P9342
TWEEN® 21	P2565
TWEEN® 40, Viscous liquid	P1504
TWEEN® 60	P1629
TWEEN® 61	P3065
TWEEN® 65	P3190
TWEEN® 80, Viscous liquid	P1754
TWEEN® 80, SigmaUltra	P8074
TWEEN® 80	P5224
TWEEN® 80, Viscous liquid, Low Peroxide	P8349
TWEEN® 80, Viscous liquid, Preservative Free, Low-peroxide, Low-carbonyls	P6474
TWEEN® 80 solution, Low Peroxide, 10% (Solution)	P0343
TWEEN® 80 solution, Liquid	P8192

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Appendix B

Detergent Product Index for Anionic, Anti-Foaming Agents, Cationic, and Zwitterionic detergents

TWEEN® 81	P8516
TWEEN® 85	P4634
Tyloxapol, SigmaUltra	T0307
Tyloxapol	T8761
n-Undecyl β-D-glucopyranoside, approx. 97% (GC)	U5254

Zwitterionic back to top

Product Name	Product #
CHAPS, minimum 98% (TLC)	C3023
CHAPS, SigmaUltra, minimum 98% (TLC)	C5070
CHAPS, for electrophoresis, minimum 98% (TLC)	C9426
CHAPSO, minimum 98%	C3649
CHAPSO, SigmaUltra	C4695
CHAPSO, for electrophoresis	C2551
3-(Decyldimethylammonio)propanesulfonate inner salt	D4266
3-(Dodecyldimethylammonio)propanesulfonate inner salt, SigmaUltra	D0431
3-(Dodecyldimethylammonio)propanesulfonate inner salt	D4516
3-(N,N-Dimethylmyristylammonio)propanesulfonate	T7763
3-(N,N-Dimethyloctadecylammonio)propanesulfonate	O8004
3-(N,N-Dimethyloctylammonio)propanesulfonate inner salt	O8638
3-(N,N-Dimethylpalmitylammonio)propanesulfonate	H6883

back to top

Appendix B

Copy of information on the Sigma-Aldrich website listing of products classified by properties of detergents. INCLUDING THE MISS CHARACTERIZATION OF TRITON X200.

SIGMA-ALDRICH

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- Peptide Explorer
- Pharmacopoeia
- Poly-Amino Acids
- Radiochemicals
- Vitamins and Derivatives

Detergent Product Index

Biological Detergents

- Anionic
- Anti-Foaming Agent
- Cationic
- Non-Ionic
- Zwitterionic

Anionic

Product Name	$
Chenodeoxycholic acid, minimum 98%	$
Chenodeoxycholic acid sodium salt, minimum 97%	$
Cholic acid, ox or sheep bile, minimum 98%	$
Dehydrocholic acid	$
Deoxycholic acid, minimum 99%	$
Deoxycholic acid, SigmaUltra, minimum 99%	$
Deoxycholic acid methyl ester, approx. 98%	$
Digitonin, Solid	$
Digitonin, cholesterol determination, approx. 50% (TLC)	$
Digitoxigenin	$
N,N-Dimethyldodecylamine N-oxide	$
Docusate sodium salt, minimum 98%, Waxy solid	$
Docusate sodium salt, SigmaUltra, minimum 99%	$
Glycochenodeoxycholic acid sodium salt, minimum 97% (TLC)	$
Glycocholic acid hydrate, synthetic, minimum 97% (TLC)	$
Glycocholic acid sodium salt hydrate, synthetic, minimum 97% (TLC)	$
Glycodeoxycholic acid monohydrate, minimum 97% (TLC)	$
Glycodeoxycholic acid sodium salt, minimum 97%	$
Glycodeoxycholic acid sodium salt, SigmaUltra, minimum 97% (TLC)	$
Glycolithocholic acid 3-sulfate disodium salt	$
Glycolithocholic acid ethyl ester	$
N-Lauroylsarcosine sodium salt, minimum 94%	$
N-Lauroylsarcosine sodium salt, SigmaUltra, minimum 97%	$
N-Lauroylsarcosine solution, minimum 95%	$
N-Lauroylsarcosine solution, 20%	$
Lithium dodecyl sulfate, for electrophoresis, denatured polyacrylamide gel electrophoresis, especially at lower temperature conditions, approx. 99% (GC)	$
Lithium dodecyl sulfate, minimum 98.5% (GC)	$
Lithium dodecyl sulfate, SigmaUltra, >99% (GC)	$
Lugol solution	$
Niaproof 4, Type 4, approx. 27%	$
Niaproof 4, SigmaUltra, Type 4, approx. 27%	$
1-Octanesulfonic acid sodium salt, SigmaUltra	$
1-Octanesulfonic acid sodium salt, approx. 98%	$
Sodium 1-butanesulfonate	$

Appendix B

Sodium 1-decanesulfonate, SigmaUltra, approx. 98%

Sodium 1-decanesulfonate, approx. 98%

Sodium 1-dodecanesulfonate

Sodium 1-heptanesulfonate anhydrous, 98%

Sodium 1-heptanesulfonate anhydrous, SigmaUltra

Sodium 1-nonanesulfonate, approx. 98%

Sodium 1-propanesulfonate monohydrate

Sodium 2-bromoethanesulfonate, minimum 98%

Sodium cholate hydrate, ox or sheep bile, minimum 99%

Sodium cholate hydrate, SigmaUltra, minimum 99%

Sodium choleate

Sodium deoxycholate, minimum 97%

Sodium deoxycholate monohydrate, SigmaUltra, >99% (titration)

Sodium dodecyl sulfate, minimum 98.5% (GC)

Sodium dodecyl sulfate, approx. 95% as based on total alkyl sulfate content

Sodium dodecyl sulfate, SigmaUltra, >99% (GC)

Sodium hexanesulfonate anhydrous, approx. 98%

Sodium hexanesulfonate anhydrous, SigmaUltra

Sodium octyl sulfate, approx. 95%

Sodium pentanesulfonate anhydrous

Sodium pentanesulfonate anhydrous, SigmaUltra

Sodium taurocholate, ox bile

Sodium taurocholate, minimum 97% (TLC)

Taurochenodeoxycholic acid sodium salt

Taurodeoxycholic acid sodium salt monohydrate, SigmaUltra, minimum 97% (TLC)

Taurodeoxycholic acid sodium salt monohydrate, minimum 97% (TLC)

Taurohyodeoxycholic acid sodium salt hydrate, minimum 98%

Taurolithocholic acid 3-sulfate disodium salt

Tauroursodeoxycholic acid sodium salt, approx. 90%

Trizma® dodecyl sulfate

Ursodeoxycholic acid, minimum 99%

Anti-Foaming Agent

Product Name

Antifoam 204

Antifoam A Concentrate

Antifoam A Emulsion

Antifoam B Emulsion

Antifoam C Emulsion

Cationic

Product Name

Alkyltrimethylammonium bromide

Benzalkonium chloride, Zephiran

Benzalkonium chloride, SigmaUltra

Benzyldimethylhexadecylammonium chloride

Benzyldimethyltetradecylammonium chloride

Benzyldodecyldimethylammonium bromide

Benzyltrimethylammonium tetrachloroiodate, minimum 98% (titration)

Dimethyldioctadecylammonium bromide

Dodecylethyldimethylammonium bromide

Appendix B

Dodecyltrimethylammonium bromide, SigmaUltra, approx. 99%
Dodecyltrimethylammonium bromide, approx. 98%
Ethylhexadecyldimethylammonium bromide
Girard's reagent T, Crystalline
Hexadecyltrimethylammonium bromide, minimum 99%, Powder
Hexadecyltrimethylammonium bromide, SigmaUltra, approx. 99%
N,N',N'-Polyoxyethylene(10)-N-tallow-1,3-diaminopropane, Liquid
Thonzonium bromide
Trimethyl(tetradecyl)ammonium bromide, approx. 99%

Non-Ionic

Product Name
BigCHAP, approx. 98%
Bis(polyethylene glycol bis[imidazoyl carbonyl]), Powder
Brij® 35, Stein-Moore chromatography
Brij® 56
Brij® 72
Brij® 76
Brij® 92V
Brij® 97
Brij® 58P
Cremophor® EL
Decaethylene glycol monododecyl ether
N-Decanoyl-N-methylglucamine, minimum 98%
n-Decyl α-D-glucopyranoside, approx. 98% (GC)
Decyl β-D-maltopyranoside, minimum 98% (GC)
n-Dodecanoyl-N-methylglucamide, approx. 98%
n-Dodecyl α-D-maltoside, minimum 98% (GC)
n-Dodecyl β-D-maltoside, minimum 98%
n-Dodecyl β-D-maltoside, SigmaUltra, minimum 98% (GC)
Heptaethylene glycol monodecyl ether, minimum 97% (GC)
Heptaethylene glycol monododecyl ether
Heptaethylene glycol monotetradecyl ether
n-Hexadecyl β-D-maltoside, minimum 98% (GC)
Hexaethylene glycol monododecyl ether
Hexaethylene glycol monohexadecyl ether
Hexaethylene glycol monooctadecyl ether
Hexaethylene glycol monotetradecyl ether
Igepal CA-630, Viscous liquid
Igepal CA-630, for electrophoresis, suitable 2-D electrophoresis
Methyl-6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside, minimum 90% (TLC)
Nonaethylene glycol monododecyl ether
N-Nonanoyl-N-methylglucamine, approx. 98%
N-Nonanoyl-N-methylglucamine, SigmaUltra, approx. 98%
Octaethylene glycol monodecyl ether
Octaethylene glycol monododecyl ether
Octaethylene glycol monohexadecyl ether
Octaethylene glycol monooctadecyl ether
Octaethylene glycol monotetradecyl ether
Octyl-β-D-glucopyranoside, minimum 98%
Pentaethylene glycol monodecyl ether, minimum 97%

Appendix B

Pentaethylene glycol monododecyl ether
Pentaethylene glycol monohexadecyl ether
Pentaethylene glycol monohexyl ether, >95% (GC)
Pentaethylene glycol monooctadecyl ether
Pentaethylene glycol monooctyl ether
Polyethylene glycol diglycidyl ether, approx. 75%
Polyethylene glycol ether W-1
Polyoxyethylene 10 tridecyl ether
Polyoxyethylene 100 stearate
Polyoxyethylene 20 isohexadecyl ether
Polyoxyethylene 20 oleyl ether
Polyoxyethylene 40 stearate
Polyoxyethylene 50 stearate
Polyoxyethylene 8 stearate
Polyoxyethylene bis(imidazolyl carbonyl)
Polyoxyethylene 25 propylene glycol stearate
Saponin from Quillaja bark
Saponin from Quillaja bark
Saponin from Quillaja bark
Span® 20
Span® 40
Span® 60
Span® 65
Span® 80
Span® 85
Tergitol, Type 15-S-12
Tergitol, Type 15-S-30
Tergitol, Type 15-S-5
Tergitol, Type 15-S-7
Tergitol, Type 15-S-9
Tergitol, Type NP-10
Tergitol, Type NP-4
Tergitol, Type NP-40
Tergitol, Type NP-7
Tergitol, Type NP-9
Tergitol, MIN FOAM 1x
Tergitol, MIN FOAM 2x
Tergitol, Type TMN-10
Tergitol, Type TMN-6
Tetradecyl-β-D-maltoside, minimum 98% (GC)
Tetraethylene glycol monodecyl ether, approx. 97% (GC)
Tetraethylene glycol monododecyl ether
Tetraethylene glycol monotetradecyl ether
Triethylene glycol monodecyl ether, minimum 97% (GC)
Triethylene glycol monododecyl ether
Triethylene glycol monohexadecyl ether
Triethylene glycol monooctyl ether, Liquid
Triethylene glycol monotetradecyl ether
Triton CF-21, Solution
Triton CF-32, 95% in water

Appendix B

Triton OF-12

Triton OF-16

Triton GR-5M, 60% in aqueous 20% isopropanol

Triton QS-15

Triton QS-44 solution, 80% in water

Triton X-100, reduced

Triton X-102

Triton X-15

Triton X-151 solution, 95% in water

Triton X-200 solution, 28% in water (dispersion)

Triton X-207

Triton® X-100, SigmaUltra

Triton® X-100, Peroxide- and carbonyl-free

Triton® X-114

Triton® X-165 solution, 70% in water

Triton® X-305 solution, 70% in water

Triton® X-405 solution, 70% in water

Triton® X-45

Triton® X-705-70 solution, 70% in water

TWEEN® 20, Viscous liquid

TWEEN® 20, Low-peroxide; Low-carbonyls

TWEEN® 20, SigmaUltra

TWEEN® 20, Low-peroxide; Low-carbonyls

TWEEN® 20 solution, 70% in water

TWEEN® 20 solution, 10% in water

TWEEN® 21

TWEEN® 40, Viscous liquid

TWEEN® 60

TWEEN® 61

TWEEN® 65

TWEEN® 80, Viscous liquid

TWEEN® 80, SigmaUltra

TWEEN® 80

TWEEN® 80, Viscous liquid, Low Peroxide

TWEEN® 80, Viscous liquid, Preservative Free, Low-peroxide; Low-carbonyls

TWEEN® 80 solution, Low Peroxide, 10% (Solution)

TWEEN® 80 solution, Liquid

TWEEN® 81

TWEEN® 85

Tyloxapol, SigmaUltra

Tyloxapol

n-Undecyl β-D-glucopyranoside, approx. 97% (GC)

Zwitterionic

Product Name

CHAPS, minimum 98% (TLC)

CHAPS, SigmaUltra, minimum 98% (TLC)

CHAPS, for electrophoresis, minimum 98% (TLC)

CHAPSO, minimum 98%

CHAPSO, SigmaUltra

CHAPSO, for electrophoresis

Appendix B

3-(Decyldimethylammonio)propanesulfonate inner salt

3-(Dodecyldimethylammonio)propanesulfonate inner salt, SigmaUltra

3-(Dodecyldimethylammonio)propanesulfonate inner salt

3-(N,N-Dimethylmyristylammonio)propanesulfonate

3-(N,N-Dimethyloctadecylammonio)propanesulfonate

3-(N,N-Dimethyloctylammonio)propanesulfonate inner salt

3-(N,N-Dimethylpalmitylammonio)propanesulfonate

Appendix C

TISSUE ENGINEERING
Volume 10, Number 11/12, 2004

OPTIMIZED ACELLULAR NERVE GRAFT IS IMMUNOLOGICALLY TOLERATED AND SUPPORTS REGENERATION

Terry W. Hudson, Ph.D., Scott Zawko, B.S., Curt Deister, B.S., Scott Lundy, Char Y. Hu, Kate Lee, and Christine E. Schmidt, Ph.D.

Appendix C

TISSUE ENGINEERING
Volume 10, Number 11/12, 2004

Optimized Acellular Nerve Graft Is Immunologically Tolerated and Supports Regeneration

TERRY W. HUDSON, Ph.D.,[1] SCOTT ZAWKO, B.S.,[1] CURT DEISTER, B.S.,[3]
SCOTT LUNDY,[2] CHAR Y. HU,[2] KATE LEE,[1] and CHRISTINE E. SCHMIDT, Ph.D.[1,2,4]

ABSTRACT

To replace the autologous graft as a clinical treatment of peripheral nerve injuries we developed an optimized acellular (OA) nerve graft that retains the extracellular structure of peripheral nerve tissue via an improved chemical decellularization treatment. The process removes cellular membranes from tissue, thus eliminating the antigens responsible for allograft rejection. In the present study, the immunogenicity and regenerative capacity of the OA grafts were tested. Histological examination of the levels of $CD8^{+}$ cells and macrophages that infiltrated the OA grafts suggested that the decellularization process averted cell-mediated rejection of the grafts. In a subsequent experiment, regeneration in OA grafts was compared with that in isografts (comparable to the clinical autograft) and two published acellular graft models. After 84 days, the axon density at the midpoints of OA grafts was statistically indistinguishable from that in isografts, 910% higher than in the thermally decellularized model described by Gulati (J. Neurosurg. 68, 117, 1988), and 401% higher than in the chemically decellularized model described by Sondell *et al.* (Brain Res. 795, 44, 1998). In summary, the results imply that OA grafts are immunologically tolerated and that the removal of cellular material and preservation of the matrix are beneficial for promoting regeneration through an acellular nerve graft.

INTRODUCTION

The majority of severed peripheral nerves are treated either by surgical realignment of the individual nerve fascicles (i.e., primary neurorrhaphy) or by implantation of an autologous nerve graft (i.e., an autograft). Primary neurorrhaphy is performed if the nerve ends can be sutured together without inducing tension; otherwise, an autograft is typically used to bridge the gap between the severed nerve ends. Development of an equally effective replacement for the autograft is needed because the procedure entails multiple surgeries and the loss of function or sensation at the donor site.[1,2] To date, no alternative as effective as the autograft at stimulating regeneration over long distances has been demonstrated.[3]

The internal structure and extracellular matrix (ECM) components of a nerve graft have been shown to be critical for guiding cell migration and nerve fiber elongation.[4–6] Thus, development of an acellular nerve graft, which contains the natural ECM components and structure but not native cells, could be valuable as an alternative to the current autograft. Acellular nerve grafts can also be used to study the roles of the ECM and cellular components concomitantly.

To create acellular grafts, the cellular components can be removed from donor tissue by several techniques in-

[1]Department of Chemical Engineering, [2]Department of Biomedical Engineering, [3]Department of Electrical Engineering, and [4]Texas Materials Institute, University of Texas at Austin, Austin, Texas.

Appendix C

cluding thermal[7] and chemical[8] processes. Thermal decellularization is the most common process in the literature; it entails subjecting the tissue to repeated freeze–thaw cycles. Although thermal decellularization does kill the cells and render the graft generally nonimmunogenic,[9,10] the process does not extract the cell remnants. As a result, an elevated number of Schwann cells and macrophages invade the basal lamina tubes to clear the cellular debris during the first days after implantation. This cellular invasion potentially delays the regenerative process and damages the basal laminae.[11–13]

Several chemical treatments have been designed to render nerve grafts nonimmunogenic while also removing much of the cellular debris. However, chemical treatments cause more damage to the ECM than thermal decellularization.[14,15] One of the most common chemical decellularization protocols in the literature was originally developed by Johnson *et al.*,[16] and later modified by Sondell *et al.*[8]

We previously developed a chemical decellularization process to create optimized acellular (OA) nerve grafts with an extracellular environment similar to that of native nerve tissue, but without the cellular material that is believed to elicit cell-mediated rejection.[17] Thorough histological evidence was presented in that article demonstrating both cell (e.g., Schwann cells) removal and ECM (e.g., basal laminae) preservation. In the present study we had two primary goals: (1) to determine whether the removal of cellular components accomplished with the OA protocol translated into an immunologically tolerated graft, and (2) to compare the regenerative capacity of the OA graft with that of other established acellular graft models. Addressing the first goal of this work, we implanted OA grafts into rats of a different strain than the donor animals (i.e., allografts). Rejection was evaluated after 28 days on the basis of the level of immune cells (e.g., T cells and macrophages) in the graft.[18] The level of cells expressing CD8$^+$ moieties (i.e., cell surface markers on cytotoxic T cells) and macrophage cells in the grafts after 28 days demonstrated that the OA grafts were not undergoing cell-mediated rejection. Thus, the removal of cellular material translated into an immunologically tolerated graft.

Regeneration in OA grafts after 28 and 84 days was compared with that in nerve grafts created according to published thermal and chemical decellularization protocols. These other models were used to study the importance of ECM preservation and cellular removal in regenerative capacity. Axon density was significantly higher in OA grafts than in the other acellular models, implying that preservation of the natural ECM and removal of cellular material are beneficial for regeneration through an acellular nerve graft. Even though regenerating nerves have been shown to grow across a 10-mm gap spontaneously, the 10-mm gap was selected for this head-to-head study. This was necessary so that our data could be compared with published work on other acellular graft models that also employed the 10-mm gap.

MATERIALS AND METHODS

Creation of grafts

To create OA grafts, both the left and right sciatic nerves were harvested under aseptic conditions from 350-g Harlan Sprague-Dawley (HSD) male rats. The tissue was handled only on the ends to minimize structural damage. On harvest, the nerves were immediately placed in RPMI 1640 solution at 4°C. All subsequent steps were conducted in a laminar flow hood for sterility. Fatty and connective tissue was removed from the nerve epineurium. The nerve tissue was cut into 15-mm segments and placed in a 15-mL conical tube filled with deionized distilled water. All washing steps were carried out at 25°C with agitation. After 7 h, the water was aspirated and replaced by a solution containing 125 mM sulfobetaine-10 (SB-10), 10 mM phosphate, and 50 mM sodium. The nerves were agitated for 15 h. The tissue was then rinsed for 15 min in a washing solution of 50 mM phosphate and 100 mM sodium. Next, the washing solution was replaced by a solution containing 0.14% Triton X-200, 0.6 mM sulfobetaine-16 (SB-16), 10 mM phosphate, and 50 mM sodium. After agitation for 24 h, the tissue was rinsed with the washing solution three times (5 min per rinse). The nerve segments were again agitated in the SB-10 solution (7 h), washed once, and agitated in the SB-16/Triton X-200 solution (15 h). Finally, the tissue segments were washed three times (15 min each) in a solution containing 10 mM phosphate and 50 mM sodium and stored in the same solution at 4°C.

Other acellular nerve graft models were created according to published methods as a basis for comparison. The chemically decellularized model was created by a protocol published by Sondell *et al.*[8] Briefly, the nerve tissue was agitated in distilled water for 7 h, in 46 mM Triton X-100 in distilled water overnight, and then in 96 mM sodium deoxycholate in distilled water for 24 h. These steps were repeated before performing a final wash in distilled water. All treatment steps were performed at room temperature, and the tissue was subsequently stored in 10 mM phosphate-buffered saline (PBS) solution at 4°C.

The thermally decellularized model (i.e., a freeze–thaw graft) was created according to the protocol described by Gulati.[19] Immediately after harvest, nerve tissue was dipped in liquid nitrogen for 20 s and thawed in PBS at room temperature for 60 s, and then the process was repeated four additional times. The freeze–thaw (F–T) grafts were placed in PBS at room temperature and used within 30 min.

Appendix C

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. All solutions were autoclaved or filter sterilized before use.

Implantation of grafts

Isografts and allografts were used to test the immunogenicity of the OA grafts. Isografts, which mimic the autograft, were harvested from a donor animal (e.g., Lewis rat) of the same strain as the host animal (e.g., Lewis rat). This served as a negative control for any immune response that results from the surgical procedure alone. Allografts were harvested from a donor animal (e.g., HSD rat) of a different strain than the host animal (e.g., Lewis rat). The differences between these rat strains are addressed in the Discussion (below). The fresh allograft served as a positive control because it is known to elicit cell-mediated rejection. The OA isograft was used to examine the *in vivo* response to the treatment protocol (e.g., response to residual chemicals). An OA allograft was inspected for residual antigens following our decellularization procedure. The four experimental conditions tested are summarized in Table 1.

Each rat was anesthetized with an intraperitoneal injection of ketamine (128 mg/kg body weight; Webster Veterinary Supply, Sterling, MA) and xylazine (15 mg/kg body weight; Webster Veterinary Supply). The sciatic nerve on the right side was exposed, transected, and 5 mm of nerve was removed. The ends of the graft were trimmed immediately before implantation to attain a clean-cut, 10-mm graft. The graft was sutured to both the proximal and distal nerve ends, using 10-0 vicryl sutures (Ethicon, Somerville, NJ). The muscle was drawn back together with 5-0 chromic gut sutures (Ethicon), and the skin was closed with wound clips (BD Diagnostics, Sparks, MD). Surgical methods were performed in accordance with regulations established by the National Research Council in the *Guide for the Care and Use of Laboratory Animals*.[30]

Immunogenicity of grafts evaluated by histology

Grafts representing all four experimental conditions were harvested 28 days after implantation. Each animal was reanesthetized, and the nerve graft was exposed. Before harvesting, the graft was fixed for 1 min with 3% glutaraldehyde–4% paraformaldehyde in PBS. The sciatic nerve was then transected 5 mm above and below the graft, the distal end was marked with a stitch, and the graft was placed in fixative at 4°C. After 30 min, the graft was transferred to PBS and stored at 4°C until it was embedded in paraffin.

Histology was used to inspect the allografts for signs of immunological rejection. The tissue was dehydrated with graded alcohol solutions and xylene, and then embedded in paraffin. Longitudinal sections of tissue, 7 μm thick, were cut with a microtome and captured on glass slides. Immunostaining was performed with anti-CD8a (BD Biosciences Pharmingen, San Diego, CA) and anti-macrophage (Chemicon International, Temecula, CA) primary antibodies. Horseradish peroxidase (HRP)-tagged secondary antibodies, 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA), and an eosin counterstain were used to visualize the invading cells. The stained sections were visualized on an Olympus IX70 (Olympus America, Melville, NY) inverted microscope, and the images were captured with an Optronics MagnaFire (Goleta, CA) digital color camera. Images of the stained tissue sections were combined in Adobe Photoshop to create a composite of the entire graft. Using Scion Image software (Scion, Frederick, MA), the percentage of area of the graft covered with positively stained $CD8^{+}$ cells and macrophages was determined.

Acellular graft models compared in vivo

To study the impact of cellular debris and structural preservation on regeneration, three acellular graft models were examined *in vivo*. OA grafts, Sondell grafts, and F–T grafts were created as described in Materials and Methods. Fresh grafts are a mimic of the clinical autograft and were included in the experiments as a positive control. The OA grafts and Sondell grafts were prepared within 30 days of implantation. The time between harvest and implantation of the F–T grafts and fresh grafts was never longer than 30 min. Donor and host animals were HSD rats.

Table 1. Implants to Examine Immunological Tolerance of Optimized Grafts

Graft type	*Donor strain*	*Host strain*	*Number of implants*	*Analyzing response to:*
Fresh isograft	Lewis[a]	Lewis	3	Surgical procedure (negative control)
	HSD[b]	HSD	3	Surgical procedure (negative control)
Fresh allograft	Lewis	HSD	3	Natural antigens (positive control)
Optimized acellular isograft	HSD	HSD	5	Treatment protocol
Optimized acellular allograft	Lewis	HSD	5	Residual antigens

[a]Lewis rats are an inbred strain (i.e., greater than 98% genetic homogeneity).
[b]HSD rats are an outbred strain, but the animals used were from a closed colony.

Appendix C

Histological comparison of decellularized tissues

A comparison of the ECM structure in the acellular grafts before implantation was conducted by visualizing the basal laminae. The grafts were prepared as previously described, embedded, and cross-sectioned. An anti-laminin primary antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) were employed in the immunostaining procedure.

Regenerative capacity of grafts evaluated by histology

Grafts were harvested 28 and 84 days after implantation (Table 2). The numbers of harvested grafts for each time point are not the same because some animals were killed early due to autotomization, which is consistent with the autotomization in HSD rats observed by others.[33]

To evaluate the regenerative potential of the three acellular graft models, longitudinal tissue sections were stained for regenerated axons, using the RT97 anti-neurofilament primary antibody (Developmental Studies Hybridoma Bank), an HRP-conjugated secondary antibody, and DAB. Subsequently, cross-sections were cut from the midpoint of the grafts and stained for neurofilaments. The stained sections were visualized with a ×20 objective and images were captured with a digital camera. A 20 × 16 cm image was printed for each sample. The number of nerve fibers in each image was counted, and the area of nerve cable in the image was measured. Because a portion of the nerve cable had been removed by sectioning the tissue longitudinally before taking cross-sections, the total number of axons in each nerve cable could not be determined. Instead, axon density was calculated by dividing the number of nerve fibers by the area of the cable from which the count was taken. Select specimens were not used in the axon density analysis if less than 33% of the nerve cable remained after longitudinal sectioning. The number of samples analyzed for each graft and time point is reported with the axon density data. Regions of connective tissue at the periphery of the graft, based on morphological evaluation, were excluded from the analysis.

Table 2. Implants to Evaluate the Regenerative Capacity of Optimized Acellular Grafts

Graft type	*Harvested (28 days)*	*Harvested (84 days)*
Fresh	9	6
Sondell	6	5
Freeze–thaw	6	4
Optimized acellular	9	6

Statistical analysis

Analysis of variance (ANOVA) was performed to determine the statistical significance of the differences between results. Specifically, an F test was used to determine whether the variability between data sets was equal or unequal. A t test was then used to determine whether the difference between the averages of the data sets was statistically significant. A significance level of $p < 0.05$ was used as the cutoff (i.e., p values are reported only for cases in which $p < 0.05$).

RESULTS

OA grafts are immunologically tolerated

To evaluate the immunological response by a host to OA grafts, four experimental conditions were tested with sciatic nerve graft implants (Table 1). By staining longitudinal sections of grafts for cytotoxic T cells and macrophages, the level of cell-mediated immune response was determined. Elevated levels of cytotoxic T cells are expected in tissues undergoing cell-mediated rejection and increased levels of macrophage cells are expected in rejected allografts. However, macrophages are also recruited during Wallerian degeneration to clear debris and release neurotrophic factors for regenerating nerves. At 28 days, both cell types could be seen throughout the full length of all grafts (Figs. 1 and 3). The infiltration of $CD8^+$ cells into fresh allografts was higher than into fresh isografts ($p < 0.01$) and OA grafts ($p < 0.005$) (Fig. 2). Meanwhile, the levels of $CD8^+$ cells in OA isografts and OA allografts were lower than those observed in fresh isografts ($p < 0.05$). Macrophage invasion into fresh isografts was lower than into fresh allografts ($p < 0.05$), but the differences between other grafts were not statistically significant (Fig. 4). Thus, histological examination of the levels of $CD8^+$ cells and macrophages that infiltrated OA grafts suggested that the decellularization process averted cell-mediated rejection of the grafts.

OA process preserves the ECM

Images of tissue sections stained for laminin allow comparison of basal laminae preservation among the decellularization protocols (Fig. 5). The ringlike structures in native nerve tissue are open columns of basal laminae (Fig. 5a), and similar structures are apparent in tissue treated according to the OA protocol (Fig. 5b) and the F–T protocol (Fig. 5c). The basal laminae appear highly fragmented in tissue treated according to the Sondell protocol (Fig. 5d).

Appendix C

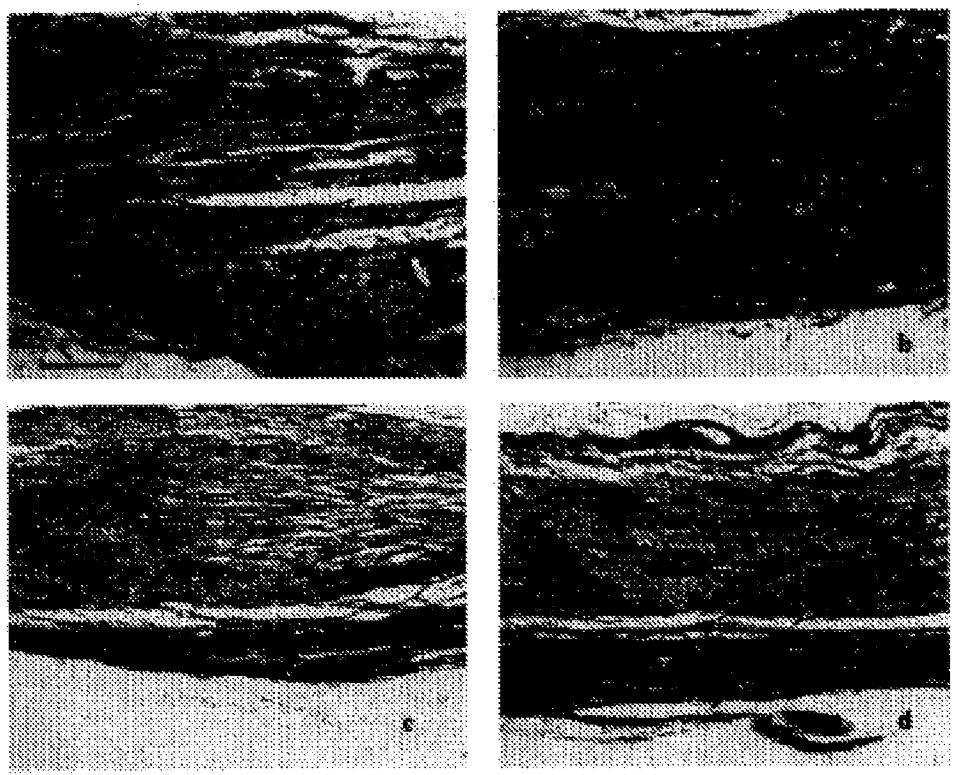

FIG. 1. Longitudinal sections of tissue were cut from (a) fresh isografts, (b) fresh allografts, (c) OA isografts, and (d) OA allografts harvested 28 days after implantation. Tissue sections were stained for CD8, a surface marker on cytotoxic T cells. The level of staining in the fresh allografts was visibly higher, but the OA grafts appeared indistinguishable from the fresh isografts. Scale bar: 200 μm.

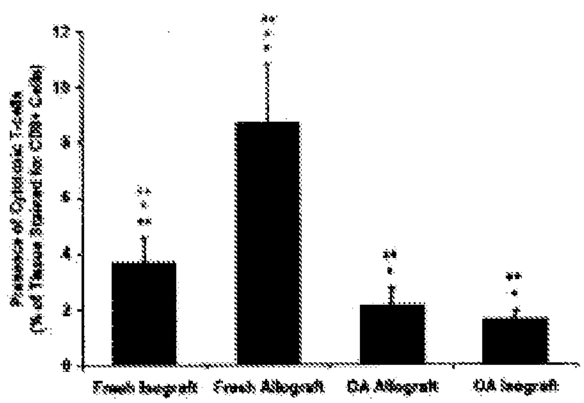

FIG. 2. Cell-mediated immune response in fresh and OA nerve grafts was evaluated by determining the percentage of tissue covered by $CD8^+$ cells. Fresh allografts demonstrated a statistically significant elevation in $CD8^+$ cells. OA isografts and allografts were statistically indistinguishable from fresh isografts, indicating that cell-mediated immune rejection was occurring only in fresh allografts. Symbols above the columns designate a significant difference from fresh isograft (*), fresh allograft (**), OA isograft (+), and OA allograft (++).

Appendix C

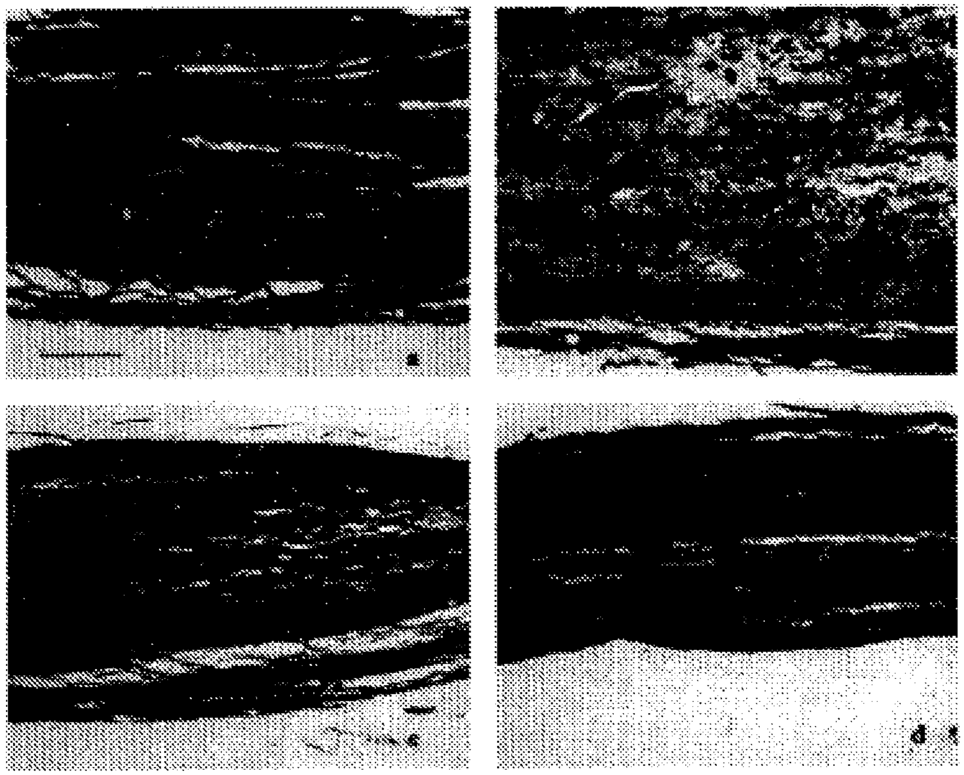

FIG. 3. Longitudinal sections of tissue were cut from (a) fresh isografts, (b) fresh allografts, (c) OA isografts, and (d) OA allografts harvested 28 days after implantation. Tissue sections were stained for macrophages, immune cells involved in Wallerian degeneration, nerve regeneration, and tissue inflammation. Scale bar: 200 μm.

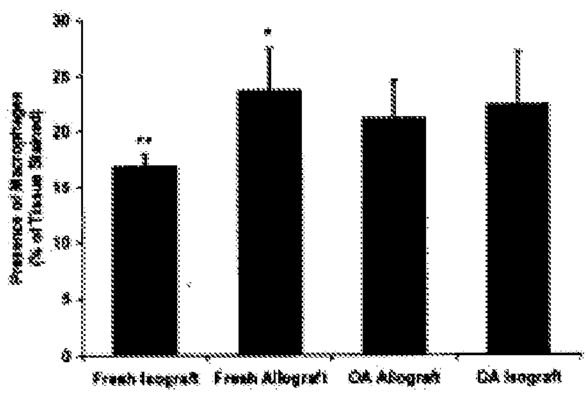

FIG. 4. Level of macrophages present in fresh and OA nerve grafts after 28 days was evaluated by determining the percentage of area stained in longitudinal tissue sections. Fresh allografts demonstrated a statistically significant elevation in macrophages compared with fresh isografts. OA isografts and allografts were statistically indistinguishable from fresh isografts, fresh allografts, and each other, suggesting that any residual chemicals in the graft did not cause a significant inflammatory response. Symbols above the columns designate a significant difference from fresh isograft (*) and fresh allograft (**).

Appendix C

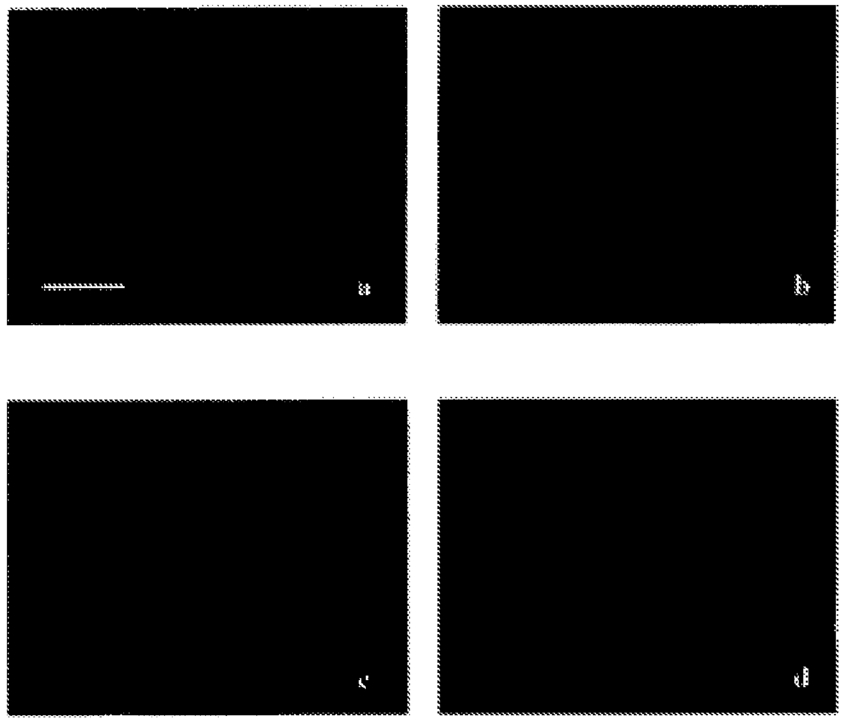

FIG. 5. Cross-sections of basal laminae were visualized by laminin staining. The ringlike appearance of the open tubes in (a) fresh nerve tissue, (b) an OA graft, and (c) an F-T graft suggests the preservation of the basal laminae. Rings are difficult to distinguish in (d) a Sondell graft, suggesting that the basal laminae were damaged during the decellularization treatment. Scale bar: 50 μm.

OA grafts support regenerating axons

The capacity of the OA graft to support nerve regeneration was tested by examining the growth of axons through the various nerve isografts after 28 and 84 days. All the grafts were isografts, harvested from and implanted into HSD rats. The grafts included (1) fresh isografts, (2) OA grafts, (3) Sondell grafts, and (4) F-T grafts. Longitudinal sections and cross-sections of the grafts were stained for neurofilaments (i.e., cytoskeletal proteins found in axons). At 28 days, new axons had grown completely across the grafts (Fig. 6). The axons appeared to meet resistance crossing from the proximal

FIG. 6. Axonal regeneration through 28-day OA nerve grafts was demonstrated by staining longitudinal tissue segments for neurofilaments. Random patterns in the axons at the junctions of the (a) proximal nerve and graft and (c) the graft and distal nerve suggest a lack of guidance as the axons crossed into and out of the graft. However, axons at (b) the midpoint of the graft were highly aligned, suggesting that they were guided by the extracellular structure of the graft. Suture marks (S) at the nerve–graft junction are shown in (a) and (c). Scale bar: 100 μm.

Application No. 10/672,689
Supp. Amdt. dated May 9, 2007
Reply to Office Action of Sept. 18, 2006

Appendix C

nerve end into the graft and from the graft into the distal nerve end, as demonstrated by the nonlinearity of neurofilaments around the suture points (Fig. 6a and 6c). However, once the axons extended into the graft, they grew linearly, as demonstrated by the parallel neurofilaments at the midpoint of the graft (Fig. 6b). Similarly, the axons grew linearly in the distal direction once they extended into the distal nerve end (data not shown). The same pattern was observed in the 84-day OA grafts. Histological staining on longitudinal sections also showed that Schwann cells were present throughout all graft types at both time points (data not shown). Thus, the OA nerve grafts supported axonal regeneration and guided axons toward the distal nerve end.

Regenerative capacity of optimized graft surpasses other acellular models

In addition to visually examining the growth of axons through the grafts, axon density in grafts was determined. The same OA grafts and fresh isografts that were harvested 28 and 84 days after implantation and sectioned longitudinally were subsequently cross-sectioned at the midpoint, stained for neurofilaments, and examined. In the 28-day grafts, the fresh grafts ($n = 9$) and OA grafts ($n = 7$) were nearly identical with axon densities of 0.9 and 0.98 axons/100 μm^2, respectively (Fig. 7). The F–T grafts ($n = 5$) had 0.50 axons/100 μm^2, and the Sondell grafts ($n = 6$) had 0.69 axons/100 μm^2. Axon density in the F–T grafts was significantly lower than in the fresh grafts and the OA grafts ($p < 0.01$). Axon density in the Sondell grafts was also significantly lower than in the fresh grafts ($p < 0.01$) and the OA grafts ($p < 0.05$).

Fresh grafts ($n = 5$) and OA grafts ($n = 5$) harvested after 84 days were still not significantly different, with axon densities of 0.73 and 0.92 axons/100 μm^2, respectively (Fig. 7). The F–T grafts ($n = 3$) had 0.10 axons/100 μm^2, and the Sondell grafts ($n = 3$) had 0.23 axons/100 μm^2. Axon density in the F–T grafts was significantly lower than in the fresh grafts ($p < 0.05$) and the OA grafts ($p < 0.05$). Axon density in the Sondell grafts was not significantly lower than in the fresh grafts, but was significantly lower than in the OA grafts ($p < 0.05$).

Because the freeze–thaw decellularization process does not remove cellular debris and the Sondell decellularization process does not preserve the ECM, the higher axon densities at 28 and 84 days in the OA grafts suggest that removing cellular debris and preserving the ECM improve the regenerative capacity of acellular nerve grafts.

DISCUSSION

An alternative method for treating severed peripheral nerves is needed to avoid multiple surgeries, donor site morbidity, and other drawbacks associated with the autograft. Acellular nerve grafts, derived from donor nerve tissue, are composed of endogenous tissue proteins. Because of their natural composition and the fact that axons preferentially grow through the basal lamina tubes

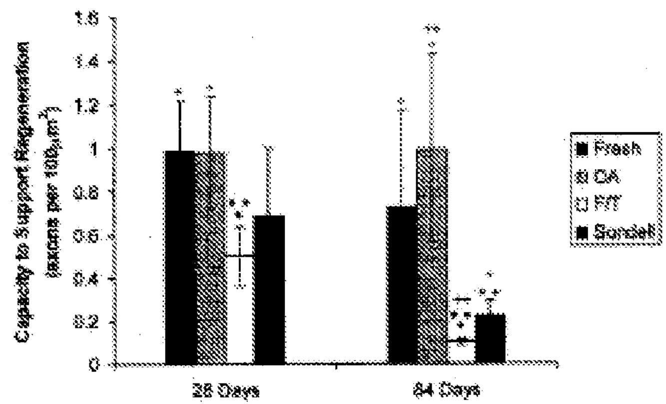

FIG. 7. The regenerative capacity of four nerve graft models was evaluated by measuring axon density in cross-sections of the grafts 28 days after implantation and 84 days after implantation. Fresh isografts served as a model for the autograft (positive control). Axon density in fresh grafts and OA grafts was statistically indistinguishable. F/T grafts had the lowest axon density, implying that the presence of cellular debris may reduce the regenerative capacity of an acellular graft. Sondell grafts demonstrated a statistically lower axon density than OA grafts after 84 days, suggesting that preservation of the ECM increased the regenerative capacity of OA grafts. Symbols above the columns designate a significant difference from fresh graft (*), OA graft (**), F/T (+), and Sondell (++) graft.

Appendix C

found in nerve tissue, acellular nerve grafts exhibit potential for use as a next-generation nerve graft. We hypothesized that improving the decellularization process to yield a better-preserved ECM would lead to an improvement in regeneration. However, for OA grafts to be used clinically, they must also be immunologically tolerated. In previous work, we developed a method of removing the cellular material believed to be responsible for immunological rejection while also preserving the ECM of nerve tissue. In the current work, the OA graft was tested *in vivo* to determine its regenerative capacity and immunogenicity.

Cellular antigens are predominantly responsible for the immunological rejection associated with nerve allografts, particularly the antigens associated with Schwann cells, endothelial cells, and macrophages.[18,22,23] The removal of cellular components by the OA protocol was correlated with the immunological response to an allograft. The major histocompatibility complex (MHC) of the rat is called RT1 and is highly polymorphic.[24] Rat strains can be characterized by their RT1 haplotype (e.g., RT1b, RT1l, RT1l). Matching of haplotypes plays a predominant role in allograft survival. Gulati and Cole demonstrated that in allografts involving strains of different RT1 haplotypes, the increased presence of immune cells associated with rejection was readily detectable at 28 days.[18] Thus, fresh nerve tissue from an HSD rat (RT1b) implanted into a Lewis rat (RT1l) (i.e., a fresh allograft) should display signs of immunological rejection after 28 days. Similarly, an acellular allograft should be rejected if the graft contains membrane-bound antigens associated with the RT1 haplotype.

Rat cytotoxic T cells carry a CD8 cell surface marker (i.e., they are $CD8^+$ cells), and the presence of cytotoxic T cells is an important indicator of cell-mediated graft rejection. However, a moderate number of $CD8^+$ cells that are not cytotoxic should be present in any nerve graft after 28 days, whether or not it is undergoing rejection. The noncytotoxic $CD8^+$ cells are a subset of macrophages that are known to invade after sciatic nerve injuries, even in the absence of rejection.[25] Macrophages are immune cells that respond to nerve injury,[26] clear cellular debris during nerve degeneration,[27] and support regeneration by inducing and producing growth factors.[28] In the case of a rejected allograft, higher numbers of macrophages should be present.[29] However, macrophages also respond to other cues in the regenerating nerve, so an increase in macrophages without a concomitant increase in $CD8^+$ cells does not indicate rejection. Thus, the presence of $CD8^+$ cells and macrophages was anticipated in all four graft models. However, a statistically significant increase in both $CD8^+$ cells and macrophages in a graft, when compared with a fresh isograft, would indicate that the graft was undergoing cell-mediated rejection.

Immunological tolerance of OA grafts was confirmed

As anticipated, the fresh allografts exhibited a statistical increase in both $CD8^+$ cells and macrophages compared with fresh isografts (Figs. 2 and 4). The OA allografts did not show an increase in $CD8^+$ cells compared with fresh isografts, indicating that they did not elicit rejection. Further evidence that the OA allografts were not rejected are the similar levels of $CD8^+$ cells and macrophages in the OA isografts and OA allografts.

Macrophage invasion into the OA grafts appeared slightly higher than invasion into the fresh isografts, although not significantly. A possible cause for the elevated level of macrophages in the OA grafts compared with the fresh isografts is that the open basal lamina tubes and the absence of myelin permitted a greater number of macrophages to invade and remain inside the OA grafts. This may be beneficial because macrophages produce growth factors. In summary, the antigens that would have initiated cell-mediated immunological rejection of OA allografts were removed.

Regenerative capacity correlated to graft structure and content

The two design criteria for the OA grafts were to remove cellular material and to provide structural support for regenerating nerves. It was hypothesized that this would improve regeneration in comparison with other acellular grafts. The importance of structural support was revealed through histological examination of longitudinal tissue sections. Axons grew linearly in regions of defined structure (e.g., in the nerve graft and distal nerve cable), but their path was irregular in regions where the graft was attached to the nerve ends (Fig. 6). The irregular patterns were potentially caused by the misalignment of basal laminae at the junctions between the nerve ends and the graft. As the axons crossed into and out of the graft, they had to find new basal laminae to provide them with guidance.

In addition to providing guidance, OA grafts also supported higher axon densities after 24 and 84 days than did other published acellular graft models (Fig. 7). The lowest axon densities were found in F–T grafts. Although the structural preservation in F–T grafts was similar to that in optimized grafts (Fig. 5), the F–T procedure was the only decellularization procedure that did not remove cellular debris. Thus, a correlation is suggested between the presence of cell debris and a reduction in the level of nerve regeneration. The primary difference between Sondell grafts and OA grafts was preservation of the ECM (Fig. 5). Consequently, the higher axon density in OA grafts suggests that providing regenerating axons with an ECM structure that mimicks native nerve is important for maximizing regeneration in an acellular graft. The im-

Appendix C

portance of these factors appears to become more evident over longer time periods, with the OA graft demonstrating axon densities 910% higher than the F-T graft and 401% higher than the Sondell graft after 84 days.

Because fresh isografts were the only grafts that contained living cells (e.g., Schwann cells and macrophages) that aid regeneration, they were expected to support higher axon densities than any of the acellular grafts. The data suggest that in 10-mm nerve grafts, the combination of desirable structure and the removal of cellular debris was sufficient to attain axon densities statistically indistinguishable from those in fresh isografts (Fig. 7). In the case of longer grafts, however, the need for support cells is expected to be more crucial. The OA graft can be used to treat injuries with longer gaps by incorporation of cells (e.g., Schwann cells) before implantation.

This work suggests that the OA graft may serve as a starting template for an off-the-shelf nerve graft. In addition, this graft is well suited for studying specific aspects of nerve regeneration. Cellular components[30,31] (e.g., Schwann cells and macrophages) and growth factors[32] are important for successful peripheral nerve regeneration. Excellent research is being performed with growth factors in fabricated systems,[33–35] but the interaction of these components with the natural nerve environment is also important and could lead to further improvements. The natural structural environment of the OA graft makes it an ideal model for studying these interactions and for examining individual cell types and growth factors through selective incorporation into the graft. As more information is gained about the role of the ECM, support cells, and growth factors, better therapeutic systems can be engineered for stimulating nerve regeneration.

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